Serum lysozyme and inflammatory bowel disease[1]

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Thirty-five patients with Crohn's disease and six patients with ulcerative colitis had elevated serum lysozyme levels compared with 47 normals, but there was no diagnostic difference in serum lysozyme levels between the two diseases. Patients with Crohn's disease were grouped according to the location of the disease: the small intestine, ileocolon, and colon. The distribution of serum lysozyme levels for normals and the distribution of levels for the three groups of patients with Crohn's disease and the group with mucosal ulcerative colitis overlapped by about one standard deviation. In addition, no substantial serum lysozyme differences were observed among the three groups of patients with Crohn's disease. Human serum lysozyme was considerably more active than the standard egg white lysozyme, and the relationship between the activities of the two lysozymes and their concentrations was linear only between 0 μg/mL and 1 μg/mL. Differences between human serum and egg white lysozyme activity may explain some of the conflicting reports in the literature. Because the manufacturer has discontinued the lysozyme turbidimetric kit, the authors provide a detailed description of the method. There were significant correlations between two lysoplate methods and between the Worthington lysoplate method and one turbidimetric method, but not between the turbidimetric method and the Kallested radial diffusion assay method.

Index terms: Colitis, ulcerative • Crohn disease • Enzyme tests • Muramidase

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A group of small (M_r 14,000–15,000), cationic (pI 10–11) enzymes with bacteriolytic activity has been named lysozyme (EC 3.2.1.17; mucopeptide N-acetyl-muramyl-hydrolase).[1–3] After Fleming first discovered lysozyme,[4]

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high concentrations were found in the kidney and in tears, serum, neutrophilic granulocytes, and mononuclear phagocytic cells.[5] Because the renal tubular transport system is quite effective,[6] only small amounts are found in the urine.[4,7–10] Lysozyme is freely filtered at the glomerulus and is reabsorbed in the proximal tubule. For this reason urinary lysozyme measurements are helpful in evaluating renal disease patients, especially kidney disease patients with tubular dysfunction.[7–13] Urinary lysozyme activity also provides a reliable method of detecting renal homograph rejection.[14]

Although increased serum lysozyme activity has been observed during the active stage of some granulomatous diseases, such as tuberculosis[15] and sarcoidosis,[16] the major use of lysozyme analysis is for hematologic disorders: markedly increased levels of lysozyme are found in the serum and urine of patients with monocytic and monomyelocytic leukemia.[17] Serum and urinary lysozyme assays are useful in distinguishing myeloid and monocytic leukemias from lymphocytic leukemia.[15,17]

In addition, lysozyme has been found to be elevated in Crohn's disease and has been used to distinguish between Crohn's disease and ulcerative colitis.[18] Other investigators were unable to confirm the elevation of lysozyme in Crohn's disease and ulcerative colitis.[19–23] One objective of this study was to reinvestigate this application of lysozyme analysis using improved methodology. Our second objective was to list the components of an enzymatic assay because a major manufacturer has discontinued making the lysozyme enzymatic kit.

Material and methods

Enzymatic method

Following are instructions for this type of assay, which is no longer available as a kit. In this method, lysozyme clears a substrate cell suspension of *Micrococcus lysodeikticus.*

Substrate

Weigh the following components: $Na_2HPO_4 \cdot 7H_2O$, 3.65 g; KH_2PO_4, 4.00 g; NaCl, 0.75 g; and 0.083 g lyophilized cells of *M lysodeikticus* (ATCC-4698) (Product number M-3770, Sigma Chemical Company, St. Louis, MO). Dissolve the substrate components in about 50 mL of distilled water, and adjust the pH to 6.0 ± 0.1 and the volume to 50 mL. Divide this preparation into 3-mL portions, and dilute each portion to 50 mL with distilled water and freeze. Before use, thaw one 50-mL portion and mix the suspension well. The substrate solution is stable for six months in the frozen state. After thawing and mixing, the substrate is stable eight hours at room temperature or 24 hours refrigerated.

Lysozyme

Weigh the following components: $Na_2HPO_4 \cdot 7H_2O$, 1.0 g and 8 mg salt-free lysozyme (Product number LS02931, Cooper Biomedical, Malvern, PA). The lysozyme should be adjusted to 100% protein. For example, if the preparation is only 80% protein, then 100%/80% × 8 would be 10 mg. Bring this mixture up to about 25 mL with distilled water, mix well, and adjust to pH 6.0 ± 0.1. Divide this solution into 1-mL aliquots and then add 7 mL of distilled water to each and freeze. This solution is stable for six months at 2–6° C and should be thawed and mixed well before use. After thawing, the standard should be refrigerated and may be used for three days.

Procedure

To make up working standards, pipet as follows into each of seven test tubes:

Standard mL	H_2O ml	Lysozyme concentration µg/mL
0.00	1.00	0
0.05	0.95	2
0.10	0.90	4
0.20	0.80	8
0.30	0.70	12
0.40	0.60	16
0.50	0.50	20

Pipet 3.0 mL of well-mixed substrate into a cuvette, and, after the reagent has reached 30° C, add 0.3 mL of standard/sample. Invert the cuvette to mix and immediately start a stopwatch. Read the absorbance change in a cuvette zeroed against a water blank between 30 seconds and 180 seconds. The rate at which the cell suspension of *M lysodeikticus* clears because of lysis is a measure of lysozyme concentration. Plot the absorbance of each standard on linear graph paper against micrograms of egg white lysozyme per

mL. The relationship is linear up to the highest standard concentration of 20 μg/mL (*Fig. 1*).

After a few analyses with human serum, it became apparent the human lysozyme was more active than the egg white lysozyme. The human serum was diluted to the range of lysozyme concentration (0–1 μg/mL) in which the relationship between absorbance and concentration remains linear (*Fig. 1*).

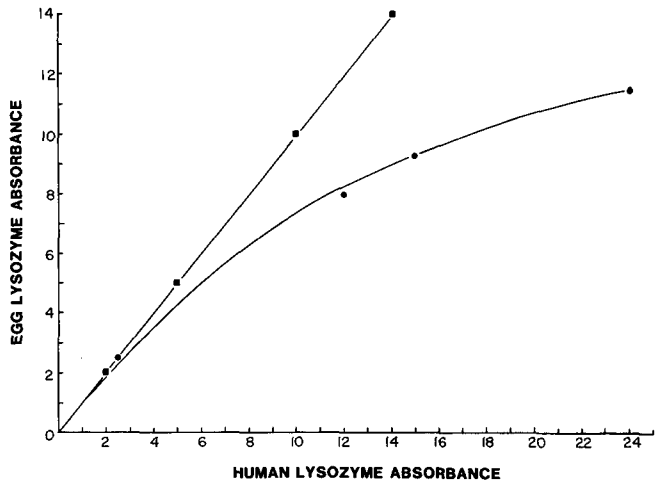

Fig. 1. Comparative absorbance of egg white lysozyme (left curve) standard curve and human serum lysozyme (right curve) diluted to linearity.

Lysoplate

Lysozyme molecules, placed in quantiplate wells (Kallestad; lysozyme quantiplate, Chaska, MN), diffuse through an agarose gel containing suspended *M lysodeikticus* cells to form a cleared zone of lysis. Each quantiplate contains up to 2.4 mL (±0.1 mL) of a pH 8.6 buffered agarose *M lysodeikticus* mixture. Each plate contains about 3.5 mg *M lysodeikticus* bacteria. A reference curve can be constructed on semilogarithmic graph paper by plotting the cleared-zone ring diameters of references on the linear axis versus their corresponding concentrations on the logarithmic axis and drawing a best-fit straight line through the adjacent points.

Even though 74-, 15-, and 3.6-μg/mL human lysozyme standards were supplied, we found a more linear standard curve could be obtained by diluting the 15 μg/mL standard (*Fig. 2*). We determined unknown concentrations were by finding the intersection of the samples' cleared-zone ring diameters with the reference curve.

Five microliters of standards or serum are placed in wells of lysoplate. The lysoplate is covered and returned to a ziploc bag, which is resealed. After incubating the lysoplate in the bag on a level surface at 23 ± 2° C, for 18 ± 0.5 hours, the cleared-zone ring diameters are measured to the nearest 0.1 mm.

We also used another type of assay plate, an experimental radial diffusion assay plate developed and used mainly at Cooper-Worthington for in-process quantitation of lysozyme. A reference egg white lysozyme solution was prepared by dissolving the enzyme standard at a concentration of 1 mg/mL in an assay buffer that was a 50 mmol/L solution of sodium phosphate, pH 7.0. The enzyme standards were diluted to concentrations of 0.5 mg/mL, 0.1 mg/mL, and 0.05 mg/mL with assay buffer. Ten microliters of each standard was added to the wells, the plates were incubated for 18 hours at room temperature, and the diameters were read to the nearest 0.2 mm (*Fig. 3*). Using semilogarithmic paper, the zone diameter for the dilution of enzyme standard was plotted in mm against the enzyme concentration on the log axis. The concentration was read using this standard curve. We also applied human lysozyme standards to the Cooper-Worthington experimental plate (*Fig. 2*) and egg white lysozyme to the Kallested lysozyme quantiplate (*Fig. 3*).

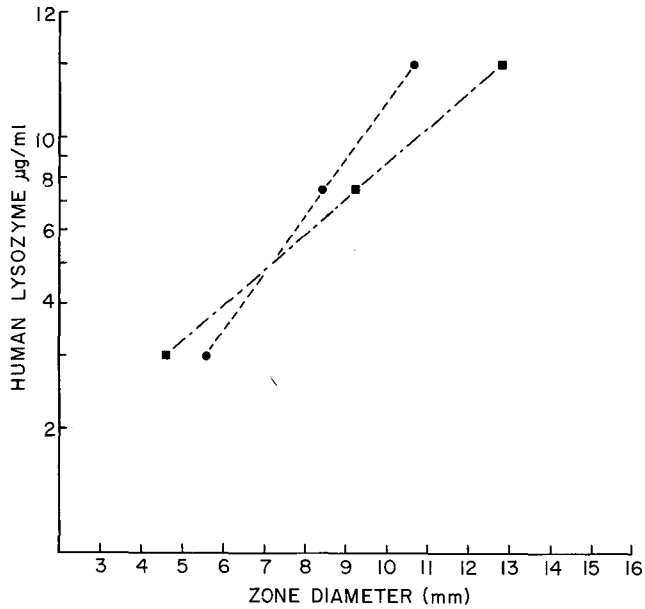

Fig. 2. Zone diameter and concentration of human lysozyme on Worthington ■——■ and ●——● Kallested lysozyme plate.

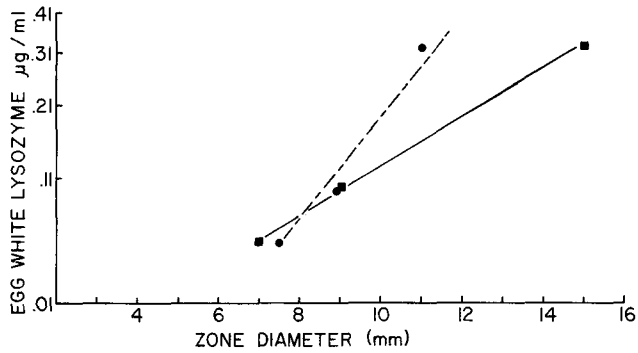

Fig. 3. Zone diameter and concentration of Worthington egg white lysozyme on Worthington ■——■ and Kallested ●——● lysozyme plate.

Patients

We measured serum lysozyme levels with the modified enzyme assay in 35 patients with Crohn's disease (14 men and 21 women, average age 34 years) and six patients with mucosal ulcerative colitis (two men and four women, average age 48 years[24]) (*Table*). In all cases, the diagnosis was confirmed by clinical, roentgenographic, and histologic criteria.[25] Ten patients had Crohn's disease of the small intestine only; 17 had ileocolitis, and eight patients had Crohn's disease that was limited to the colon. We determined serum lysozyme levels by comparing the patients' results directly with the egg white lysozyme curve and by diluting the serum to linearity on the egg white lysozyme curve (*Fig. 1*). We also measured lysozyme levels in 55 hospital patients with other than gastrointestinal disease with the enzymatic method, the lysoplate from Kallested, and the experimental radial diffusion assay plate from Cooper-Worthington. To determine the in-run variation, we ran the same sample 25 times by the same enzyme method.

Results

The Table shows the results read directly from the egg lysozyme graph, and matched results read from another graph after the serum was diluted to linearity (*Fig. 1*), for patients with Crohn's disease and with mucosal ulcerative colitis as well as for normals. The values read from the graph using the egg white lysozyme standard were low and did not show large differences among the groups. However, higher values and marked differences were observed between Crohn's disease, ileitis, ileocolitis, and colitis and mucosal ulcerative colitis patients and normals ($P < 0.01$). We could not distinguish between Crohn's disease and mucosal ulcerative colitis based only on serum lysozyme levels. Forty-seven normals had a mean and standard deviation of 30.4 ± 13.8 µg/mL and a normal range (2 SD) of 13–58 µg/mL. Overlap, averaging close to one standard deviation, exists between the normal distribution and the three groups with Crohn's disease and the group with mucosal ulcerative colitis. Twenty-five consecutive analyses of the same sample had a mean of 48.8 ± 4.1 (SD) µg/mL (CV = 8.4%). Twenty-five samples run on a day-to-day basis had a mean of 47.4 ± 4.5 (SD) µg/mL (CV = 9.5%).

The curve for egg white lysozyme concentration versus absorbance was linear to 20 µg/mL (*Fig. 1*). However, diluting human serum showed that human lysozyme was much more active than the egg white lysozyme. The range in which both human lysozyme activity and egg white activity were linear was 0–1 µg/mL. In some cases it was necessary to dilute the sera several times before the range of 0–1 µg/mL was attained. However, similar values could be found by diluting the sera until a value was reached that could be found on a human lysozyme calibration curve.

Table. Lysozyme activity in sera from normals and from patients with Crohn's disease and mucosal ulcerative colitis

Bowel Disease	No. of patients	Enzyme activity	
		Regular egg white*	Human lysozyme†
Normals	47	13.6 ± 2.8 µg/mL	30.4 ± 13.8 µg/mL
Crohn's disease	35	14.6 ± 2.0 µg/mL	65.1 ± 10.1 µg/mL
Ileitis	10	15.4 ± 2.1 µg/mL	72.0 ± 11.0 µg/mL
Ileocolitis	17	14.4 ± 1.9 µg/mL	64.3 ± 10.2 µg/mL
Colitis	8	14.3 ± 2.0 µg/mL	58.2 ± 8.8 µg/mL
Mucosal ulcerative colitis	6	14.3 ± 3.1 µg/mL	56.5 ± 10.8 µg/mL

* Compared directly with an egg white lysozyme curve (*Fig. 1*) and assuming linearity.
† Diluted linearity on the egg white lysozyme curve.

When the sera from the 55 hospital patients were assayed, the correlation coefficient between the Kallested lysoplate method and the Cooper-Worthington enzymatic lysozyme kit was 0.133 ($P > 0.05$). In contrast, the correlation coefficient between the Worthington radial diffusion assay plate and the Cooper-Worthington enzymatic lysozyme method was 0.31 ($P < 0.05$). The correlation coefficient of 0.65 ($P < 0.001$) between the two radial diffusion assays from Kallested and Worthington was much greater.

Discussion

Our finding that serum lysozyme is elevated in patients with inflammatory bowel disease agrees with the findings of Falchuk et al,[18] but, unlike them, we were unable to distinguish between Crohn's disease and mucosal ulcerative colitis solely on the basis of serum lysozyme levels. Using the turbidimetric method, Hylander et al[20] also observed elevated serum lysozyme levels in both patients with ulcerative colitis and patients with Crohn's disease, but there was much overlap between the two groups. In contrast, when we used the lysoplate method, there were no significant differences between the normals and the groups of patients with ulcerative colitis and Crohn's disease. Ayulo et al,[23] using a lysoplate method, and Peeters et al[22] and Nugent et al,[21] using a turbidimetric method, found an elevation of lysozyme in about half of their patients with Crohn's disease. Normals and patients with ulcerative colitis had about the same levels. Using the lysoplate method, Sells and Carpenter[19] reported no differences between the normals and patients with either ulcerative colitis or Crohn's disease.

The turbidimetric method may be less diagnostically useful than the lysoplate method because of the assumed linear relationship between lysozyme concentration and absorbance in the turbidimetric method. The turbidimetric assay using egg white lysozyme is likely used to place a value on the lysoplate controls. In our experiment with the lysoplate method controls, the controls had no relationship to one another, and we took one control and diluted it two different ways to establish the human lysozyme value. For example, in the turbimetric assay an absorbance of 11 for egg white lysozyme activity is about equivalent to an absorbance of 20 for the same concentration of human lysozyme, but an absorbance of 16 for egg white lysozyme is equivalent to an absorbance of about 55 for the same concentration of human lysozyme (*Fig. 1*). Therefore, incorrect lysoplate values could have been derived from the turbidimetric method. Other inherent problems between a plate or mass assay and the turbidimetric method may also exist.

The differential diagnosis between Crohn's disease and ulcerative colitis can be made with certainty in about 95% of cases. However, there is a sufficient overlap of clinical, radiological, and histological characteristics to make differentiation difficult in about 5% of the patients.[25] A differential diagnosis is important because of the risk of carcinoma in patients with mucosal ulcerative colitis. However, we could not confirm the finding of Falchuk et al[18] that lysozyme analysis can provide a differential diagnosis between Crohn's disease and mucosal ulcerative colitis. Instead, we found elevations of lysozyme in both diseases.

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